

Heat shock factor 1 regulates lifespan as distinct from disease onset in prion disease

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Prion diseases are fatal, transmissible, neurodegenerative diseases caused by the misfolding of the prion protein (PrP). At present, the molecular pathways underlying prion-mediated neurotoxicity are largely unknown. We hypothesized that the transcriptional regulator of the stress response, heat shock factor 1 (HSF1), would play an important role in prion disease. Uninoculated HSF1 knockout (KO) mice used in our study do not show signs of neurodegeneration as assessed by survival, motor performance, or histopathology. When inoculated with Rocky Mountain Laboratory (RML) prions HSF1 KO mice had a dramatically shortened lifespan, succumbing to disease $\approx 20\%$ faster than controls. Surprisingly, both the onset of home-cage behavioral symptoms and pathological alterations occurred at a similar time in HSF1 KO and control mice. The accumulation of proteinase K (PK)-resistant PrP also occurred with similar kinetics and prion infectivity accrued at an equal or slower rate. Thus, HSF1 provides an important protective function that is specifically manifest after the onset of behavioral symptoms of prion disease.

neurodegeneration | HSF1 | transmissible spongiform encephalopathy | PrP | protein misfolding

At present there are few treatments and an incomplete understanding of the pathogenic mechanisms at play in neurodegenerative diseases. Prion diseases are a special class of protein-misfolding disorders, in that they can be transmitted within and sometimes between species in addition to occurring in sporadic and inherited forms (1). In prion diseases the host-encoded, normally folded, prion protein (termed PrP^C) is converted into a rogue self-perpetuating conformation (termed PrP^{Sc}) (1, 2). The ongoing conversion of PrP^C to an infectious conformer, PrP^{Sc}, is an absolute requirement for neurotoxicity in prion disease (3, 4), yet beyond this basic observation little is known about the cellular events leading to synaptic loss and neuronal death.

Protein-folding homeostasis, or “proteostasis” (5), is maintained by two evolutionarily conserved pathways: the unfolded protein response (UPR) and the heat shock response (HSR). The UPR is induced when the protein-folding machinery within the endoplasmic reticulum (ER) is saturated with unfolded or misfolded proteins (6). To relieve the ER protein-folding burden, the UPR reduces the synthesis of secretory proteins while also up-regulating ER protein-folding machinery, such as ER chaperones (6).

The second protein-folding homeostatic mechanism is the HSR. The HSR was first characterized in terms of the organismal responses to extreme changes in environmental temperatures and stresses (7–9). It has since been determined that the HSR is the primary means for cells to cope with misfolded proteins in the cytosol (10). Under normal conditions, the heat shock transcription factor (HSF) is sequestered in the cytosol in a complex with molecular chaperones (11, 12). During conditions that perturb cytosolic protein-folding homeostasis, the chaperones are diverted elsewhere. Then HSF trimerizes and enters the nucleus (13, 14), where it rapidly drives transcription of numerous genes involved in protein refolding, degradation of misfolded proteins, and other proteins that regulate stress tolerance (15). In yeast and *Caeno-*

rhabditis elegans there is only one HSF gene whereas in mammals there are HSF1, HSF2, and HSF4. Although interplay between HSFs is complex (16), HSF2 and HSF4 have tissue restricted roles, such as in spermatogenesis and lens formation (17–20). The general picture emerging from gene targeting studies in mice defines HSF1 as the critical global responder to stress (21).

The relevance of the HSR and molecular chaperones to diseases involving protein misfolding is a topic of intense interest (5, 10, 22, 23). Indeed, several animal models of neurodegeneration treated with heat shock inducing compounds show delayed disease symptoms and pathology (24–26). In a prion disease affecting humans, Creutzfeldt–Jakob disease, a prominent induction of heat shock proteins was noted in the Purkinje cells of the cerebellum (27). Also, up-regulation of HSF1 target genes such as polyubiquitins and Hsp70 genes was observed in a mouse model of infectious prion disease (28). Because prion diseases are an extreme example of diseases of protein misfolding, we asked whether HSF1 regulated prion pathogenesis.

We used mice deficient for HSF1 (21) to address the causality of the HSF1-mediated HSR in protecting against prion disease. Under basal conditions, HSF1 knockout (KO) mice in our colony have a very subtle hypomyelination phenotype but show no signs of neurodegenerative disease at a neuropathological or behavioral level. When challenged with prions, however, HSF1 KO mice die considerably faster than control mice. Surprisingly, the behavioral changes associated with prion disease are initiated at the same time in HSF1 KO and control mice, as are neuropathological alterations. Proteinase K (PK)-resistant PrP accumulates at a similar rate in the brains of prion-infected HSF1 KO and control mice, and prion infectivity, as determined by bioassay, is equal or slightly diminished at several time points after inoculation in HSF1 KO brains. We have uncovered a protective role for HSF1 in prion pathogenesis and establish that it is specific to disease progression as distinct from disease onset.

Results

Characterization of Neurological Parameters in Uninoculated HSF1 Knockouts. Previously, HSF1 KO mice were characterized in terms of (1) heat shock protein induction in response to high tempera-

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The authors declare no conflict of interest.

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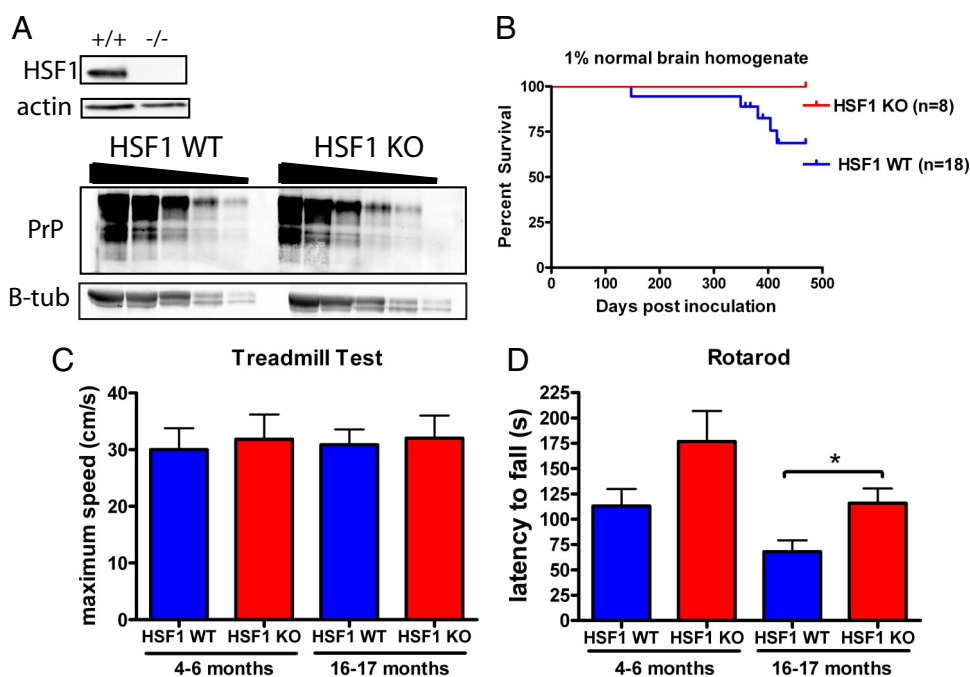
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Fig. 1. Characterization of uninfected HSF1 KO mice. (A) Western blots verifying the absence of HSF1 from a whole brain homogenate from a genotypically HSF1 null mouse. Dilution series of whole brain homogenates from HSF1 WT ($n = 2$) and HSF1 KO ($n = 2$) show similar steady-state levels of PrP. Beta-tubulin was used as a loading control. (B) Assessment of survival in HSF1 WT and KO mice inoculated with a 1% normal brain homogenate (from hamster): 69% of HSF1 WT mice ($n = 18$) survived up to 469 days postinoculation when the study was terminated whereas 100% of HSF1 KO mice survived ($n = 8$) (tick marks indicate intercurrent deaths or censored events, such as mice killed because of extreme dermatitis or from fight-related injuries). (C) Motor performance was assessed on an accelerating treadmill (acceleration: 1 cm/s per 5 seconds). The maximum speed at which mice could run was recorded at 4–6 months ($n = 7$ WT and $n = 6$ HSF1 KO) and 16–17 months ($n = 8$ for each group); there were no significant differences between HSF1 WT and KO mice at either time point. Error bars represent the SEM. (D) Motor coordination was measured on the rotarod apparatus for HSF1 WT ($n = 7$) and HSF1 KO ($n = 6$) at 4–6 months of age and at 16–17 (*, $P = 0.02$, Student's *t* test). Error bars represent the SEM.



tures, (2) systemic inflammation, (3) neonatal lethality, and (4) cardiac ischemia. In the present study, we analyzed whether PrP protein expression was affected by deletion of HSF1, because the *Prnp* gene has heat shock elements in its promoter, which may promote its expression in response to elevated temperature (29). Western blots of a 2-fold dilution series of whole brain homogenates (1–0.06%) from HSF1 WT and HSF1 KO show similar steady-state levels of PrP (Fig. 1A).

It was also reported that HSF KO mice have abnormalities in their brains (16, 30). Santos and colleagues noted that their HSF1 KO brains had enlarged ventricles and astrogliosis, particularly in white matter regions (30), while Homma and colleagues also identified diminished myelination in HSF1 KO mice and motor deficiencies (16). Thus, we conducted detailed neuropathological analysis of the HSF1 KO mice from our breeding colony, which is maintained on a mixed 129SV \times BALB/c \times C57BL/6 background, to determine whether these phenotypes were present. In brains taken from HSF1 WT and KO mice that were either uninoculated or mock inoculated intracranially (IC) with 1% normal brain homogenate (an inflammatory stimulus) we detected no evidence of neurodegeneration. We did note a subtle white matter defect in the cerebellum and sometimes in the corpus callosum of HSF1 KO brain visualized with hematoxylin and eosin staining, which showed a variable amount of vacuolation [one representative sample is shown in [supporting information \(SI\) Fig. S1](#)]. This phenotype was also present in HSF1 WT mice but at a much lower frequency. Using the Luxol-Nissl myelin stain there was a slightly weaker reaction in the HSF1 KO brain sections ([Fig. S1 A and B](#)). GFAP staining, a measure of gliosis, was present to a variable degree in this region of the cerebellum of HSF1 KO mice although not altogether absent from HSF1 WT samples ([Fig. S1 A and B](#)). Staining for IBA1, a microglial marker, and CNPase, an enzyme expressed in myelinating cells, did not show a phenotypic difference in HSF1 WT and KO samples (data not shown and [Fig. S1](#)).

To determine the functional significance (if any) of this subtle, restricted white matter defect, we conducted several experiments. First, we assessed the survival of HSF1 WT and KO mice inoculated IC with a 1% normal hamster brain homogenate: 69% of HSF1 WT

mice survived up to 469 days postinoculation (DPI) when the study was terminated whereas 100% of HSF1 KO mice survived (Fig. 1B). This result suggests that HSF1 KO mice have a normal lifespan at least beyond the latest time points examined in our study of prion-infected animals.

Motor performance was assessed on an accelerating treadmill as conducted previously (31). There were no significant differences between HSF1 WT and KO mice at 4–6 or 16–17 months of age (Fig. 1C). Motor coordination was measured on the rotarod apparatus (32). Surprisingly, HSF1 KO mice showed a trend toward better performance on rotarod at 4–6 months old, remaining on the accelerating rotarod for ≈ 60 seconds longer than HSF1 WT mice ($P = 0.082$, Student's *t* test) (Fig. 1D). At a late time point of 16–17 months of age, HSF1 KO mice outperformed HSF1 WT mice on the rotarod ($P = 0.020$, Student's *t* test). Because the rotarod tests motor function that relies heavily on cerebellar function and other phenotypic testing did not show defects in HSF1 KO mice, we concluded that these mice do not suffer from detectable neurodegeneration under basal conditions. This suggests that previous reports of degenerative phenotypes in HSF1 KO mice are because of genetic modifiers used in the backgrounds. Indeed HSF1 KO mice may provide a means to uncover natural genetic variation that could contribute to white matter pathologies.

Survival of Prion-Inoculated HSF1 Knockouts. Next HSF1 WT and KO mice were inoculated with the Rocky Mountain Laboratory Chandler (RML) strain of murine adapted scrapie prions. When injected directly into the brain, RML prions (4.5logLD₅₀/30 μ l) caused HSF1 KO mice to die 18% faster than littermate HSF1 WT controls measured by median survival times. The median survival time for HSF1 KO mice was 165 DPI ($n = 15$) and HSF1 WT mice was 200 DPI ($n = 18$) ($P < 0.0001$, log rank test) (Fig. 2A). We inoculated separate cohorts of mice with a range of doses of RML prions IC and observed a shortening of disease course at all doses tested in the HSF1 KO mice compared to HSF1 WT control mice (Table S1). We also administered prions via an i.p. route to test whether there was a differential peripheral prion replication or neuroinvasion phenotype in HSF1 KO mice compared to HSF1

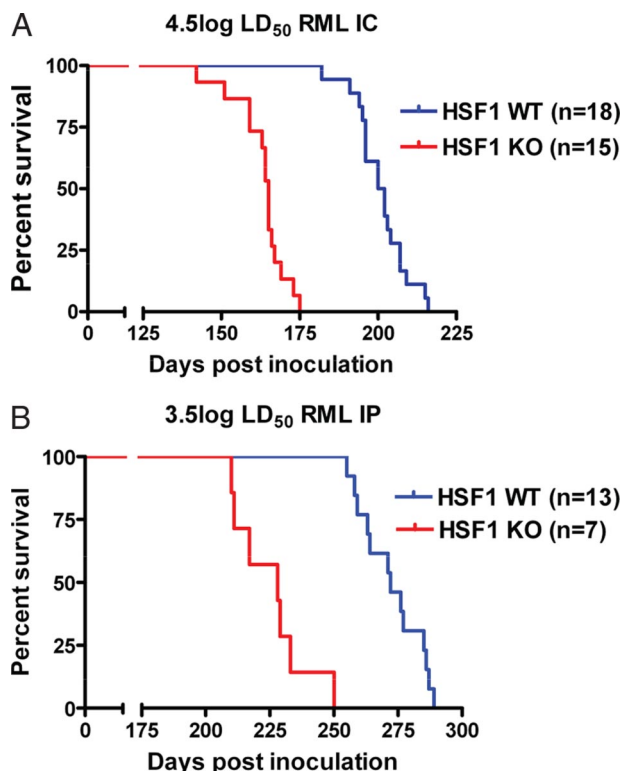


Fig. 2. HSF1 KO mice die faster than HSF1 WT control mice from prion disease. (A) Survival of HSF1 WT ($n = 18$) and KO ($n = 15$) mice inoculated intracranially with 4.5logLD₅₀ RML prions ($P < 0.0001$, log rank test) (note: x-axis is broken to begin at 125 days postinoculation). (B) i.p. infection of HSF1 WT ($n = 13$) and KO ($n = 7$) mice with 3.5logLD₅₀ RML prions ($P < 0.0001$, log rank test) (note: x-axis is broken to begin at 175 days postinoculation).

WT mice. HSF1 KO mice ($n = 7$) inoculated with 3.5logLD₅₀ RML IP succumbed to disease faster than HSF1 WT control mice ($n = 13$) by 44 days (Fig. 2B and Table S1) ($P < 0.0001$, log rank test). Because the difference between IP and IC inoculations with 3.5logLD₅₀ RML prions was quite similar ($\approx 16\%$ enhancement of disease in IP inoculated mice and $\approx 18\%$ enhancement in IC inoculated mice), there is unlikely to be a change in peripheral prion replication or neuroinvasion in HSF1 KO mice compared to HSF1 WT mice. We also noted that homozygous wild-type (HSF1^{+/+}) and heterozygote HSF1 knockouts (HSF1^{+/-}) had identical disease progression. We therefore combine HSF1^{+/+} and HSF1^{+/-} mice as “HSF1 WT” in this study.

Onset of Prion Disease Behavioral Symptoms in HSF1 Knockouts. To determine at what stage HSF1 exerts its protective effect, we first investigated the alterations in home-cage behaviors of a cohort of RML-infected HSF1 WT and KO mice. We used video-based automated behavioral analysis technology, which robustly discriminated between mock-inoculated control mice and RML prion-infected mice at the earliest onset of disease (33). This system was able to distinguish subtle differences in prion-induced behavioral symptoms in another study (34). We followed the behavioral changes in RML prion-inoculated HSF1 WT ($n = 20$) and HSF1 KO ($n = 15$) mice (from survival plot shown in Fig. 1) beginning at 1 month postinoculation (MPI) until death.

Given that the HSF1 KO mice died >1 month faster than control mice, we expected to observe a commensurate quickening in the onset and spectrum of behavioral signs of neurological disease. Surprisingly, the onset of behavioral symptoms was quite similar or only slightly accelerated in HSF1 KO mice compared to littermate HSF1 WT mice (Fig. 3).

One of the most obvious phenotypes that results from RML prion disease is a dramatic increase in activity, which is reflected in the lateral distance traveled in the home cage (33). The distance traveled in a 24-hour recording period began to increase in HSF1 WT and KO mice at 4 MPI and became much more pronounced by 4.5 and 5 MPI (Fig. 3A). Although the HSF1 KO mice show a trend toward increasing distance traveled at 4 and 4.5 MPI, there were no significant differences from HSF1 WT mice. Another phenotype of prion disease, increased rearing, shows a similar increase in HSF1 KO and control mice except for the last recording time point of HSF1 KO mice, 5 MPI. Here the HSF1 KO mice rear less than HSF1 WT controls ($P < 0.01$, Wilcoxon rank sum for all home-cage behavioral statistical tests) (Fig. 3B). A decrease in hanging vertically or upside down from the wire food rack of the home cage, a complex motor behavior, is characteristic of RML prion disease (33). This decrease in hanging showed a similar progressive decline in both HSF1 WT and KO mice. At one early time point, 2.5 MPI, HSF1 KO mice actually exhibited a significant increase in hanging ($P < 0.05$) and at the last recording of HSF1 KO mice, 5 MPI, there was a significant decrease in the hanging behavior ($P < 0.05$) (Fig. 3C). Decreased grooming is another feature of RML prion infection (33). This progressive decline in grooming behavior was slightly more severe in HSF1 KO mice than in HSF1 WT mice ($P < 0.05$ at 4.5 and $P < 0.01$ at 5 MPI) (Fig. 2D).

Analysis of several other behaviors of HSF1 WT and KO mice, including jumping, resting, stretching, and drinking were indistinguishable with the exception of eating, which was significantly decreased at many time points in HSF1 KO mice (data not shown). Thus, our analysis suggests that the onset of RML prion-induced behavioral signs of neurological disease occurs at a similar time point (≈ 4 –4.5 MPI) in HSF1 KO mice compared to control mice. However, the clinical course—the length of time from the onset of disease phenotypes until death—is drastically reduced in HSF1 KO mice, hence their absence from the later behavioral time points.

Neuropathological Features of Prion Disease in HSF1 Knockouts.

Neuropathological analysis of brain samples taken from IC prion-inoculated HSF1 KO and control mice were conducted at several time points: 3.5, 4, 4.5 MPI and at endpoint. Hematoxylin and eosin (H&E) stained sections were assessed for spongiform changes in prion-inoculated samples. At 3.5 MPI there was little to no spongiosis. At 4 and 4.5 MPI, and in terminal samples, vacuolation was evident in both prion-inoculated HSF1 WT and KO brain samples (Fig. 4). The rate of progression, extent, or regional distribution of vacuolation did not differ between HSF1 WT and KO samples.

The amount of gliosis, an established marker for neurodegeneration, was assessed by staining brain sections with an antibody against the astrocyte antigen, glial fibrillary acidic protein (GFAP). Abundant anti-GFAP staining was evident in all prion-inoculated samples by 4–4.5 MPI. As with H&E staining observations, we did not note any differences in the regional distribution, strength, or kinetics of the glial response (Fig. 4). We stained for PrP aggregates by treating brain samples with formic acid to diminish the normal endogenous PrP, leaving only disease-associated aggregated PrP deposits. There were no differences in PrP aggregate accumulation in the brains of HSF1 KO and control samples (Fig. 4).

Accumulation of Proteinase K-resistant PrP and Prion Infectivity.

Because immunohistochemical staining for PrP aggregation is not quantitative, we used PK digestion to assay the amount of aggregated PrP and to distinguish disease-associated PrP from normal PrP, which is sensitive to digestion with PK. We measured the amount of PK-resistant PrP in whole brain homogenates from prion-inoculated HSF1 WT and HSF1 KO mice killed at 3.5 MPI and also in samples taken from terminally ill mice. At 3.5 MPI, PK treatment (50 μ g/ml for 1 h at 37°C) digested away almost all of PrP, but a faint amount of PK-resistant PrP was visible in almost all samples, irrespective of the HSF1 genotype (Fig. 5A). In terminal

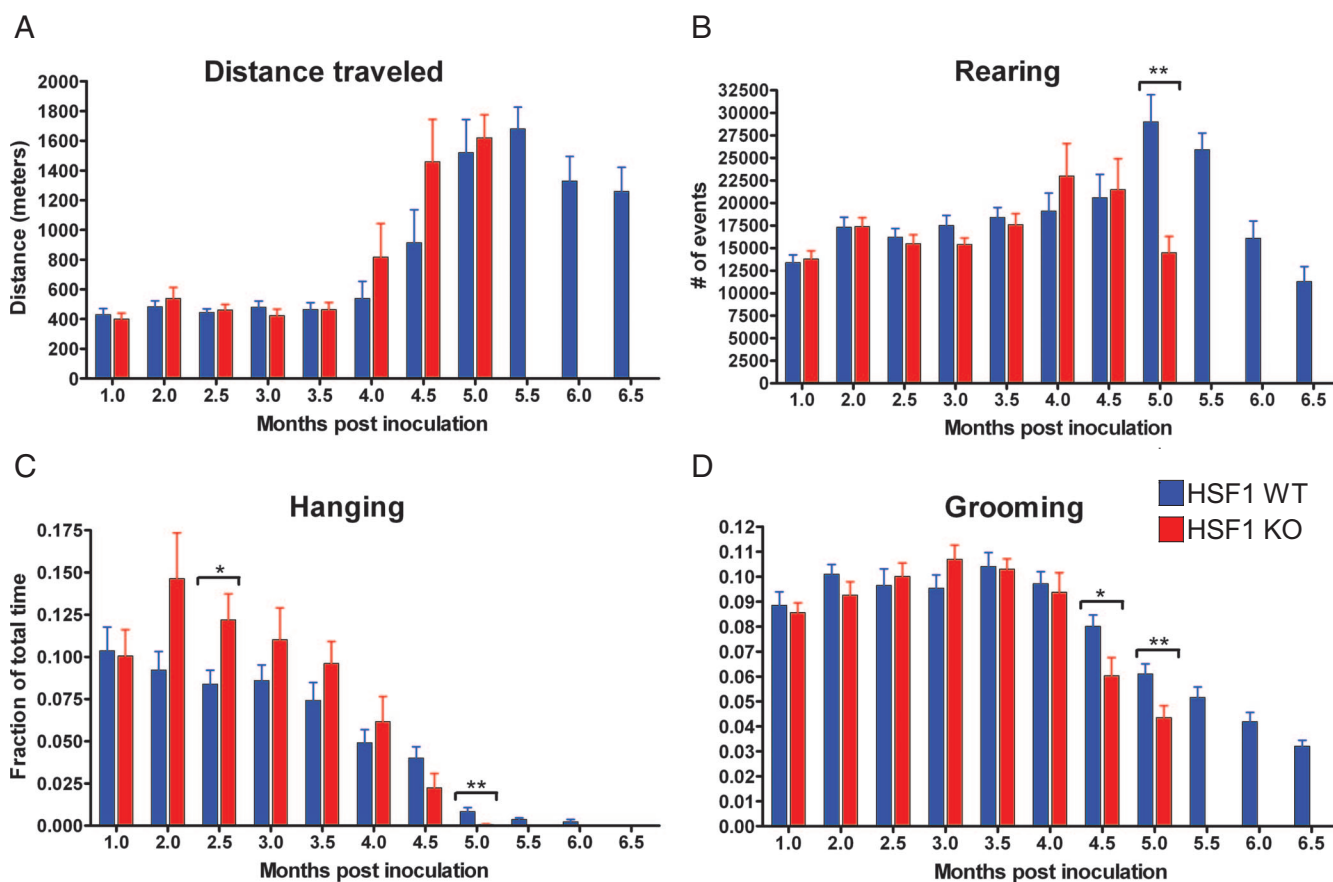


Fig. 3. Behavioral alterations in home-cage behaviors occur at a similar time in prion-infected HSF1 WT and KO mice (for HSF1 WT mice $n = 20$ at all time points except at 6.5 MPI where $n = 19$ and for HSF1 KO mice $n = 15$ at all time points except at 5 MPI where $n = 14$). (A) The increase in distance traveled, which measures lateral movement (meters), shows a similar onset in HSF1 WT and KO mice infected with RML prions. (B) Another phenotype of prion disease, increased rearing, shows a similar increase in HSF1 KO and control mice except for the last recording time point of HSF1 KO mice, 5 MPI, where the HSF1 WT mice showed an increased rearing ($P < 0.01$, Wilcoxon rank sum). (C) The decrease in hanging from the wire food rack was similar between HSF1 WT and KO mice (at one early time point, 2.5 MPI, HSF1 KO mice had a significant increase in hanging $P < 0.05$, Wilcoxon rank sum test) and at the last recording of HSF1 KO mice, 5 MPI, there was a significant decrease in the hanging behavior of HSF1 KO mice ($P < 0.05$, Wilcoxon rank sum). (D) The decrease in grooming behavior was slightly more severe in HSF1 KO mice, which showed less grooming at 4.5 ($P < 0.05$) and 5 MPI ($P < 0.01$, Wilcoxon rank sum test). HSF1 KO mice were not video recorded beyond 5 MPI because many had already died. Error bars represent the SEM.

samples there was considerably more accumulation of PK-resistant PrP in all HSF1 WT and HSF1 KO samples (Fig. 5B).

Because PK-resistance does not always predict prion infectivity (35, 36), we determined prion titers using a bioassay (37). We inoculated a 10-fold dilution series of HSF1 WT and HSF1 KO brains from 2, 3, 4, and 5 MPI into “Tga20” transgenic mice overexpressing WT mouse PrP (38) and monitored their survival. We also assayed prion infectivity in terminal brain samples from prion-inoculated HSF1 WT and HSF1 KO samples using CD1 mice as recipients. Unexpectedly, we observed a similar or even diminished development of prion infectivity in HSF1 KO mice relative to HSF1 WT mice (Fig. 5C, Table S2). At 2 MPI, there was a small but nonetheless statistically significant decrease in titer ($P = 0.019$, P -value from bootstrap resampling) and in terminal samples there was also a small but significant decrease in prion titer ($P = 0.016$) (Fig. 5C, Table S2). There were no statistically significant differences in prion titers between HSF1 KO and HSF1 WT mice at the other time points tested (3, 4, and 5 MPI).

Discussion

We have demonstrated that HSF1 is a key regulator of lifespan in prion disease. Importantly, the other major features of prion disease—the onset of behavioral symptoms, neuropathological changes, and aggregation of PrP—show similar timing in HSF1 KO

and control mice. The disease symptoms of RML prion inoculation occur ~4 MPI in both the HSF1 WT and KO mice, with some subtle changes, such as decreased grooming, happening only slightly earlier in the HSF1 KO mice. The accumulation of PK-resistant PrP was observed first at 3.5 MPI in both the HSF1 WT and KO brain samples and prion titers showed a parallel increase over the course of disease. Thus HSF1 KO mice have a much shorter postsymptomatic phase of disease than WT mice, defining a critical protective activity by HSF1 as operating after disease phenotypes first arise.

How might HSF1 function to prolong the survival of prion-infected mice? There are two major arms in the cellular response to protein-folding stress. Whereas the UPR responds primarily to protein-folding stress in the secretory pathway (6), the HSF1-mediated HSR responds to protein-folding stress in the cytosol and nucleus (10). Interestingly, the cytosol has been implicated as a key compartment for PrP toxicity by cell culture and *in vivo* experiments (39). It may be that in the absence of the HSF1 transcriptome, neurons cannot protect themselves against the accumulation of PrP in the cytosol during prion infection.

HSF1 also functions as a transcriptional repressor for another set of target genes and some studies directly link this repressor activity to apoptosis (40, 41). At present there is conflicting evidence on the importance of apoptotic pathways in prion diseases (34, 42, 43).

netically defined crossing of transgenic lines. Numerous reports have noted up-regulation of particular chaperones that correlate with neuroprotection (48–50), but by taking a genetic approach we were able to demonstrate that HSF1 is required for maintaining viability during the clinical phase of prion infection. Indeed, boosting the HSR during prion disease may be an effective therapeutic strategy. Previous studies demonstrated that prion-infected neuroblastoma cells present a defective heat shock response (51). Although the mechanism of this effect remains unclear, it was later shown that geldanamycin, a potent heat shock-inducing drug, restores this defective heat shock response in cultured cells infected with prions (52). Activation of HSF1 might provide a promising therapeutic strategy for ameliorating prion or other protein-misfolding diseases. However, there are good reasons to be cautious about induction of the HSR because HSF1 has diverse target genes that are involved in many cellular processes (15) such as maintenance of circadian rhythms (53) and promoting tumor cell maintenance (22). Therefore, the design of therapeutics with a high specificity for induction of the HSR in neurons accumulating misfolded proteins may be an important avenue for treating protein-misfolding disorders.

Materials and Methods

Mouse Strains. All mouse experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care. Food and water were provided ad libitum and mice were singly housed for the duration of the study while being maintained on a 12:12 light–dark cycle. The construction of the HSF1 deletion (54) and characterization of the knockout mice (21) were previously described. Mice were obtained on a mixed 129SvEv × BALB/c strain background and were crossed once to C57BL/6J and maintained by intercrossing mice heterozygous for the HSF1 deletion allele. The titering of the infectivity of samples used Tga20 recipient mice that had been backcrossed to C57BL/6J for 5–6 generations and bred onto a WT PrP genome (55).

The detailed methods of prion inoculations and titer calculation, statistical analysis, Western blotting, behavioral analysis, and neuropathological analysis are described in *SI Methods*.

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